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Phylogenetic Analysis of Nepenthaceae, Based on Internal Transcribed Spacer Nuclear Ribosomal DNA Sequences

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Nepenthaceae, a monotypic family of carnivorous pitcher plants comprising *Nepenthes*, is widely distributed in Southeast Asia. To determine the phytogeography of *Nepenthes* in Southeast Asia, and to trace the evolutionary trends of taxonomically important characteristics (i.e., peristomes) of the genus, we analyzed 57 internal transcribed spacer (ITS) nuclear ribosomal DNA (nrDNA) sequences of 56 species of *Nepenthes* and 1 ITS sequence each of *Dionaea muscipula* and *Ancistrocladus robertsoniorum*. To clarify the phylogenetic relationships of *Nepenthes*, we examined four different methods of phylogenetic tree reconstruction. The resulting tree topologies were mostly consistent with one another except for the basal polytomies. Seven monophyletic subclades could be recognized. Similarities and differences in terms of the positions of taxa between the present study and previous studies were observed. Judging from the phylogenetic trees and distribution area of each species, Borneo appears to be a secondary center of diversification for *Nepenthes* and species of *Nepenthes* may have then radiated within the Sunda Shelf of Southeast Asia. The three character states of the peristomes from the upper pitchers were relatively well correlated with the grouping of the species of *Nepenthes* within seven subclades and showed the limitations of the Danser (1928) system for *Nepenthes*.

Key words: ITS, *Nepenthes*, phylogenetic relationship, phytogeography, peristome

Nepenthaceae, a monotypic family of carnivorous pitcher plants comprising *Nepenthes*, is widely distributed in the Asian tropics, mainly in Southeast Asia and the Sunda Shelf region. The vast majority of species grow in moist regions throughout the Old World tropics, as far west as Madagascar (*N. madagascariensis* and *N. masoalensis*), the Seychelles (*N. pervillei*), and Sri Lanka (*N. distillatoria*) to India (*N. khasiana*) in the north, Australia (*N. tenax* and *N. rowanae*) in the south; and New Caledonia (*N. vieillardii*) in the east (Clarke 2007, Krutzsch 1988, McPherson 2009).

The genus comprises 120 species in Southeast Asia, especially Sumatra (37 species, 29 endemic), Borneo (36 species, 29 endemic), and the Philippines (21 species, 20 endemic). Although most extant species of *Nepenthes* are distributed to the west of the Wallace line, some species, such as *N. ampullaria*, *N. gracilis*, *N. mirabilis*, and *N. tentaculata*, occur in both Asia and Wallacea (McPherson 2009). The distribution of the genus has been attributed to biogeographic factors occurring both recently and in the past, including the connection of the Sunda Shelf islands caused by sea level drop and the isolation of the islands caused by increased sea levels, changes in global climate, and the ability of the species of *Nepenthes* to disperse and colonize new habitats (Clarke 2006, Danser 1928, McPherson 2009, Meimberg *et al.* 2001).

Meimberg *et al.* (2001) performed a molecular

phylogenetic analysis of *Nepenthes* based on DNA sequences of the plastid *trn*K intron. The *trn*K intron is more variable than *rbc*L (Johnson & Soltis 1995, van den Berg *et al.* 2005) and is widely used for reconstruction of lower taxonomic levels and has therefore been the locus of choice in molecular phylogenetic analyses of Nepenthaceae (Meimberg & Heubl 2006). The results of their study assumed that colonization of Southeast Asia by *Nepenthes* was initiated from India, since the Indian endemic, *N. khasiana*, was a sister taxon to all Asian taxa of *Nepenthes*. A comparative analysis between the *Nepenthes trn*K intron and its translocated copy, however, has demonstrated a topological incongruence. Meimberg *et al.* (2006) and Meimberg & Heubl (2006) pointed out the possibility of introgression or lineage sorting as the reason for the topological incongruence of the phylogenetic trees. Meimberg & Heubl (2006) then introduced PTR1 (peptide transferase 1), a nuclear low copy gene as a phylogenetic marker, but the resolution of the phylogenetic tree was low and some taxa appeared in different positions from the previous findings in the tree topology. Meimberg *et al.* (2001) provided two possible interpretations concerning the origin of *Nepenthes*; evolution in the northern Tethys, or a Gondwanan origin at a time when the Indian plate was separated from Madagascar. From the molecular analysis based on *mat*K, they suggested that colonization of Southeast Asia was from an ancient Indian stock and subsequently a new secondary center of diversity developed in the Malay Archipelago. Re-evaluation of the origin and diversification of *Nepenthes* using another gene is needed because of topological incongruence and low resolution of the phylogenetic trees obtained from previous studies.

In our study, we used nucleotide sequences of the internal transcribed spacer (ITS) to resolve phylogenetic relationships within *Nepenthes*. The characteristics of the ITS region, with its small size, highly conserved flanking regions, and fast evolutionary rate, have made this nuclear ribosomal DNA (nrDNA) sequence a valuable marker for phylogenetic analysis (Baldwin *et al.* 1995). In addition, ITS sequences from *N. ventricosa* and *N. alata* have been studied to evaluate the potential value of ITS for phylogenetic reconstruction. This study implies that the ITS regions of these species have many variable characteristics that are potentially informative for resolving the phylogeny of *Nepenthes* (Alejandro *et al.* 2008). Ribosomal DNA genes, however, are present in high copy numbers and may therefore be subjected to directional concerted evolution (Wendel *et al.* 1995a) or intergenomic introgression (Wendel *et al.* 1995b).

The objectives of this study were to clarify the phylogenetic relationships of *Nepenthes* based on ITS nucleotide sequences for 1) determining the phytogeography of *Nepenthes* in Southeast Asia and to re-evaluate the scenario suggested by Meimberg *et al.* (2001), and 2) trace the evolutionary trends of peristomes in the genus. Peristomes are stiff structures comprising an inwardly curved rim surrounding the pitcher opening (McPherson 2009). Peristomes have been used in taxonomic studies (Danser 1928) to distinguish between related species (Cheek & Jebb 2009, Lee *et al.* 2006, McPherson 2009, Robinson *et al.* 2009) and to determine new species (Cheek & Jebb 2009, Lee *at al.* 2006, Robinson *et al.* 2009).

Materials and Methods

Plant materials

We analyzed 57 samples from 56 species of *Nepenthes* representing all geographical areas (Table 1). Of the included species, seven (*N. chaniana*, *N. lingulata*, *N. mindanaoensis*, *N. naga*, *N. platychila*, *N. thai*, and *N. vogelii*) were found within the last 10 years (Akhriadi *et al.* 2009, Cheek & Jebb 2009, Lee *et al.* 2006, McPherson 2009) and have never been used in phylogenetic studies. Two samples from distantly distributed *N. mirabilis* were also examined. The first (*N. mirabilis*1) was from Bengkulu, Sumatra, and the second (*N. mirabilis*2) was from West Kalimantan, Borneo. The seven species and the two distantly distributed samples of *N. mirabilis* from the Malay Archipelago were expected to contribute to a better understanding of the phytogeogra-

phy of *Nepenthes* in Southeast Asia. *Ancistrocladus robertsoniorum* (Ancistrocladaceae, Genbank: GQ443551) and *Dionaea muscipula* (Droseraceae, Genbank: AB675913) were used as outgroups, because these two families have been recognized as sister groups to Nepenthaceae on the basis of macromolecular characteristics using nuclear 18S rDNA and plastid *rbc*L, *atp*B, and *mat*K sequences (Albert *et al.* 1992, Cuenoud *et al.* 2002, Hilu *et al.* 2003).

Amplification and sequencing

Total DNA was extracted from silica gel-dried leaf samples with a Qiagen DNeasy Mini Plant Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Amplification of the ITS region from Ancistrocladaceae was performed using a set of primers, AITS1 (5'-AGAAGTCCACTGAACCTTATC-3') and AITS4 (5'-CGCTTCTCCAGACTACAATTC-3'), which are angioseperm specific and do not co-amplify fungal DNA (Meimberg *et al.* 2010). The amplification reaction for the ITS region included Ex-Taq buffer and Ex-Taq DNA polymerase (Takara Bio, Shiga, Japan). The polymerase chain reaction (PCR) protocol consisted of an initial 90 s predenaturation at 96°C; 30 cycles of 20 s at 96°C (denaturation), 40 s at 57°C (annealing), and 40 s at 72°C (extension); and a final 7 min extension at 72°C. For seven species samples (*N. ampullaria*, *N. hirsuta*, *N. rowanae*, *N. danseri*, *N. neoguineensis*, *N. papuana*, and *N. tentaculata*), annealing temperature was changed to 58.5°C.

The PCR products were cleaned using the Wizard SV Gel and PCR Clean Up System (Promega) and were used for autocycle sequencing reactions following the manufacturer's (Beckman Coulter) instructions. Autocycle sequencing products were cleaned by ethanol precipitation. Both forward and reverse sequences were analyzed with a CEQ8000 automated sequencer (Beckman Coulter), using the same primers as for PCR. A set of internal primers, AITS2R (5'-TGCGTTCAAAGACTCGATGG-3') and AITS3F (5'-GAAGAACGTAGCGAAATGCG-3'), was designed to achieve better analysis of the ITS region. The sequence of the ITS region from each sample was used for phylogenetic analysis; all sequences were deposited in DDBJ/EMBL/Genbank (Table 1).

Phylogenetic analysis

DNA sequences obtained from the ITS region were aligned with ClustalX (Larkin *et al.* 2007). Phylogenetic analysis involving the maximum parsimony (MP) method was performed using the PAUP (Phylogenetic Analysis using Parsimony) program, version 4.0b10 (Swofford 2002). Data were analyzed by the heuristic search method with the tree bisection-reconnection (TBR) branch swapping and MulTrees options on and stepwise addition with simple addition sequences using one reference taxon (*N. thai*). All of the most parsimonious trees (MPTs) were saved. All characters were equally weighted and unordered (Fitch 1971). Gaps were treated as missing data. To evaluate the internal support of clades, bootstrap analysis (Felsenstein 1985) was conducted using 1000 replicates in a heuristic search with the TBR branch swapping and MulTrees options off. The number of steps, consistency indices, and retention indices (Farris 1989) were calculated with one of the MPTs in each analysis using the TREE SCORES command in PAUP*. For comparison, we also performed phylogenetic analysis using three different methods.

(1) Bayesian analysis with MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) by using GTR+I+G model selected from the Modeltest (Posada 2008) as the optimum model for sequence evolution based on AIC criterion. Four chains were run for 1,000,000 generations and were sampled every 100 generations to yield a posterior probability distribution of 10,000 trees. The first 2500 trees were discarded as burn in. To check whether the MCMC chain was long enough to reach convergence, the trace files resulting from the Bayesian analysis were opened in the Tracer program (v1.5) (Rambaut & Drummond 2007), and their effective sample size (ESS) statistics were calculated for values higher than 100.

(2) The neighbor joining (NJ) method (Saitou & Nei 1987) with MEGA version 5.05 (Tamura *et al.* 2011) by using the Tamura 3-parameter model

TABLE 1. Plant materials examined in this study. The materials were collected from the Indonesian Carnivorous Plant Society (*Komunitas Tanaman Karnivora Indonesia/KTKI*), Bogor Botanical Garden (BBG, Indonesia), and Kyoto Botanical Gardens (KBG, Japan). All specimens were deposited in TI (*FA: Firman Alamsyah).

Species	Source	Voucher Specimens*	Voucher No. of living stocks	Genbank accession no. of ITS
Nepenthes adnata Tamin & M. Hotta ex Schlauer	KTKI	FA-110001	B111109 01	AB675864
Nepenthes alata Blanco	KTKI	FA-110002	C14110901	AB675865
Nepenthes alba Ridl.	KTKI	FA-110003	E05021001	AB675866
Nepenthes ampullaria Jack	BBG	FA-110049	KRB27100901	AB675914
Nepenthes bellii K. Kondo	KTKI	FA-110004	A111109 01	AB675868
Nepenthes burbidgeae Hook. f. ex Burb	KTKI	FA-110005	B14110901	AB675869
Nepenthes burkei Mast.	KTKI	FA-110006	E05021002	AB675870
Nepenthes campanulata Sh. Kurata	KTKI	FA-110007	B14110902	AB675871
Nepenthes chaniana C. Clarke *et al.*	KTKI	FA-110008	B11110902	AB675872
Nepenthes clipeata Danser	KTKI	FA-110009	D07110901	AB675873
Nepenthes copelandii Merr. ex Macfarlane	KTKI	FA-110010	E05021003	AB675874
Nepenthes danseri Jebb & Cheek	KTKI	FA-110050	A14110901	AB675915
Nepenthes densiflora Danser	KTKI	FA-110057	C14110902	AB675875
Nepenthes diatas Jebb & Cheek	KTKI	FA-110011	D071109 02	AB675876
Nepenthes distillatoria L.	KTKI	FA-110012	E05021004	AB675877
Nepenthes ephippiata Danser	KTKI	FA-110013	A14110902	AB675878
Nepenthes faizaliana J.H. Adam & Wilcock	KTKI	FA-110014	E05021005	AB675879
Nepenthes fusca Danser	KTKI	FA-110015	A141109 03	AB675880
Nepenthes glabrata J.R. Turnbull & A.T. Middleton	KTKI	FA-110016	A11110902	AB675881
Nepenthes gracilis Korth.	BBG	FA-110017	KRB27100902	AB675882
Nepenthes hirsuta Hook. f.	KTKI	FA-110051	D07110903	AB675916
Nepenthes khasiana Hook. f.	KTKI	FA-110018	E05021006	AB675883
Nepenthes lingulata Chi. C. Lee, Hernawati & Akhriadi	KTKI	FA-110019	A11110903	AB675884
Nepenthes longifolia Nerz & Wistuba	KTKI	FA-110020	E05021007	AB675885
Nepenthes macrovulgaris J.R. Turnbull & A.T. Middleton	KTKI	FA-110021	B14110903	AB675886
Nepenthes madagascariensis Poir.	KBG	FA-110058	KBG02-0521	AB769064
Nepenthes merrilliana Macfarlane	KTKI	FA-110022	A11110904	AB675887
Nepenthes mindanaoensis Sh. Kurata	KTKI	FA-110023	A141109 04	AB675888
Nepenthes mirabilis (Lour.) Druce (Bengkulu, Sumatra)	KTKI	FA-110024	E05021008	AB675889
Nepenthes mirabilis (Lour.) Druce (West Kalimantan)	KTKI	FA-110025	E05021009	AB675890
Nepenthes naga Akhriadi *et al.*	KTKI	FA-110026	D07110904	AB675891
Nepenthes neoguineensis Macfarlane	KTKI	FA-110052	A14110905	AB675917
Nepenthes ovata Nerz & Wistuba	KTKI	FA-110027	B11110903	AB675892
Nepenthes papuana Danser	KTKI	FA-110053	B141109 04	AB675918
Nepenthes pervillei Blume	KTKI	FA-110028	E05021010	AB675893
Nepenthes platychila Chi. C. Lee	KTKI	FA-110029	E05021011	AB675894
Nepenthes rajah Hook. f.	KTKI	FA-110030	D07110905	AB675895
Nepenthes reinwardtiana Miq.	BBG	FA-110031	KRB27100903	AB675896
Nepenthes rhombicaulis Sh. Kurata	KTKI	FA-110032	B141109 05	AB675897
Nepenthes rowanae F.M. Bailey	KTKI	FA-110054	A14110906	AB675919
Nepenthes sanguinea Lindl.	KTKI	FA-110033	B14110906	AB675898
Nepenthes smilesii Hemsl.	KTKI	FA-110034	C14110903	AB675899
Nepenthes spathulata Danser	KTKI	FA-110035	D07110906	AB675900
Nepenthes spectabilis Danser	KTKI	FA-110036	A14110907	AB675901
Nepenthes sp. Misool	KTKI	FA-110037	E05021012	AB675902
Nepenthes stenophylla Mast.	KTKI	FA-110038	A141109 08	AB675903
Nepenthes sumatrana (Miq.) Beck	KTKI	FA-110039	A14110909	AB675904
Nepenthes talangensis Nerz & Wistuba	KTKI	FA-110040	D07110907	AB675905
Nepenthes tentaculata Hook. f.	KTKI	FA-110055	C141109 04	AB675920
Nepenthes thai Cheek	KTKI	FA-110041	E05021013	AB675906
Nepenthes tobaica Danser	KTKI	FA-110042	B11110904	AB675907
Nepenthes truncata Macfarlane	KTKI	FA-110043	E05021014	AB675908
Nepenthes veitchii Hook. f.	KTKI	FA-110044	A14110910	AB675909
Nepenthes ventricosa Blanco	KTKI	FA-110045	A11110905	AB675910
Nepenthes vieillardii Hook. f.	KBG	FA-110059	KBG02-0522	AB769065
Nepenthes villosa Hook. f.	KTKI	FA-110046	B14110907	AB675911
Nepenthes vogelii Schuit. & de Vogel	KTKI	FA-110047	B11110905	AB675912
Dionaea muscipula J. Ellis ex L.	KTKI	FA-110048	E05021015	AB675913

(Tamura 1992). Bootstrap values were calculated with 1000 replicates.

(3) The maximum likelihood (ML) method with the Treefinder program (Jobb *et al.* 2004) by using GTR+G model, which was suggested based on AIC criterion by the "propose model" analysis implemented in the Treefinder program. Bootstrap values were calculated with 1000 replicates.

Reconstruction of character states

To study the phytogeography of *Nepenthes* in Southeast Asia, we mapped the character states for distribution areas onto one of the MPTs by using the MacClade program (v4.06), with accelerated character transformation (ACCTRAN) optimization (Maddison & Maddison 2003). In addition, we also mapped the character states for the peristome of upper pitchers to study the evolutionary trends of morphological characteristics of *Nepenthes*. The morphometric data of the peristomes of the upper pitchers were taken from McPherson (2009).

Upper pitchers are distinguishable from lower pitchers. They are produced from leaves along a climbing stem of older plants of *Nepenthes*, in which the tendril attaches to the back of the pitcher on the side where the lid meets the rear of the pitcher orifice. In contrast, the lower pitchers, which are developed from a tendril that attaches to the front of the pitcher on the same side as the orifice, are produced by young plants of *Nepenthes* (Di Giusto *et al.* 2008, Gaume & Di Giusto 2009, McPherson 2009, Moran 1996). All but one of the species of *Nepenthes* examined in this study showed this pitcher dimorphism. *Nepenthes campanulata* produces only one type of pitcher. We included data for the peristomes of these pitchers, since they develop from leaves farther up the stem of mature plants and show tendril attachment as in the typical upper pitchers of *Nepenthes*. We classified peristomes as having three character states: narrow (<10 mm), intermediate (10–20 mm), and broad (>20 mm).

Results

Molecular phylogeny of Nepenthes

We obtained 57 new ITS nrDNA sequences of 56 species of *Nepenthes* and 1 ITS sequence of *Dionaea muscipula*. The alignment of 59 entire ITS sequences (57 ingroup sequences plus 2 outgroups) provided an 856-bp-long matrix. Sequence length variations resulting from insertions and deletions were found among the species of *Nepenthes*. The aligned ITSs contained 341 (39%) constant characters, 239 (29%) parsimony-uninformative variable characters, and 276 (32%) parsimony-informative characters. The analysis resulted in 324 MPTs with a length of 1130 steps and had consistency (CI) and retention (RI) indices of 0.665 and 0.687, respectively. The strict consensus tree reconstructed by the parsimony method is shown in Fig. 1. The trees obtained from the NJ, ML, and Bayesian analysis methods were mostly consistent with the tree obtained from the parsimony method, except for basal species, which appeared in different positions (Fig. 1 & Fig. 2). We compiled the bootstrap values of MP, NJ, ML, and posterior probabilities of Bayesian analysis on the MP tree (Fig. 1).

Using *Ancistrocladus robertsoniorum* and *Dionaea muscipula* as outgroups, we recognized two basal branches and seven subclades (I–VII) in the phylogenetic tree (Fig. 1). In addition, we recognized a basal group consisting of seven species whose branching pattern could not be resolved well. The seven subclades (I–VII) were supported by all four phylogeny reconstruction methods, except for subclade I. The members of subclade I were grouped with some of the basal unresolved species in the Bayesian tree (Fig. 2). Corresponding to the tree topology, the two basal taxa were *N. pervillei* from Seychelles and *N. madagascariensis* from Madagascar. Subclade I comprised two species from outlying areas (India (*N. khasiana*) and Sri Lanka (*N. distillatoria*)); a species from New Guinea (*N. papuana*), and a species (*N. ampullaria*) distributed across Peninsular Malaysia, Sumatra, Borneo, and New Guinea. Subclade II consisted of a species endemic to Sulawesi (*N. glabrata*), a species from Sulawesi and Borneo (*N. tentaculata*), and a species endemic to Borneo (*N. hirsuta*). Subclade III contained species restricted to the Philippines and a

species endemic to Borneo (*N. campanulata*) in the basal position. Subclade IV also contained species restricted to the Philippines. Subclade V contained only species from Borneo. Subclade VI comprised exclusively four species from Peninsular Malaysia and Indochina (*N. smilesii*, *N. sanguinea*, *N. alba*, and *N. thai*), a species endemic to Borneo (*N. macrovulgaris*), and a species occuring in Borneo and Sumatra (*N. reinwardtiana*) in the basal position. Subclade VII comprised 13 species of *Nepenthes* from Sumatra and one species (*N. spathulata*) also distributed in

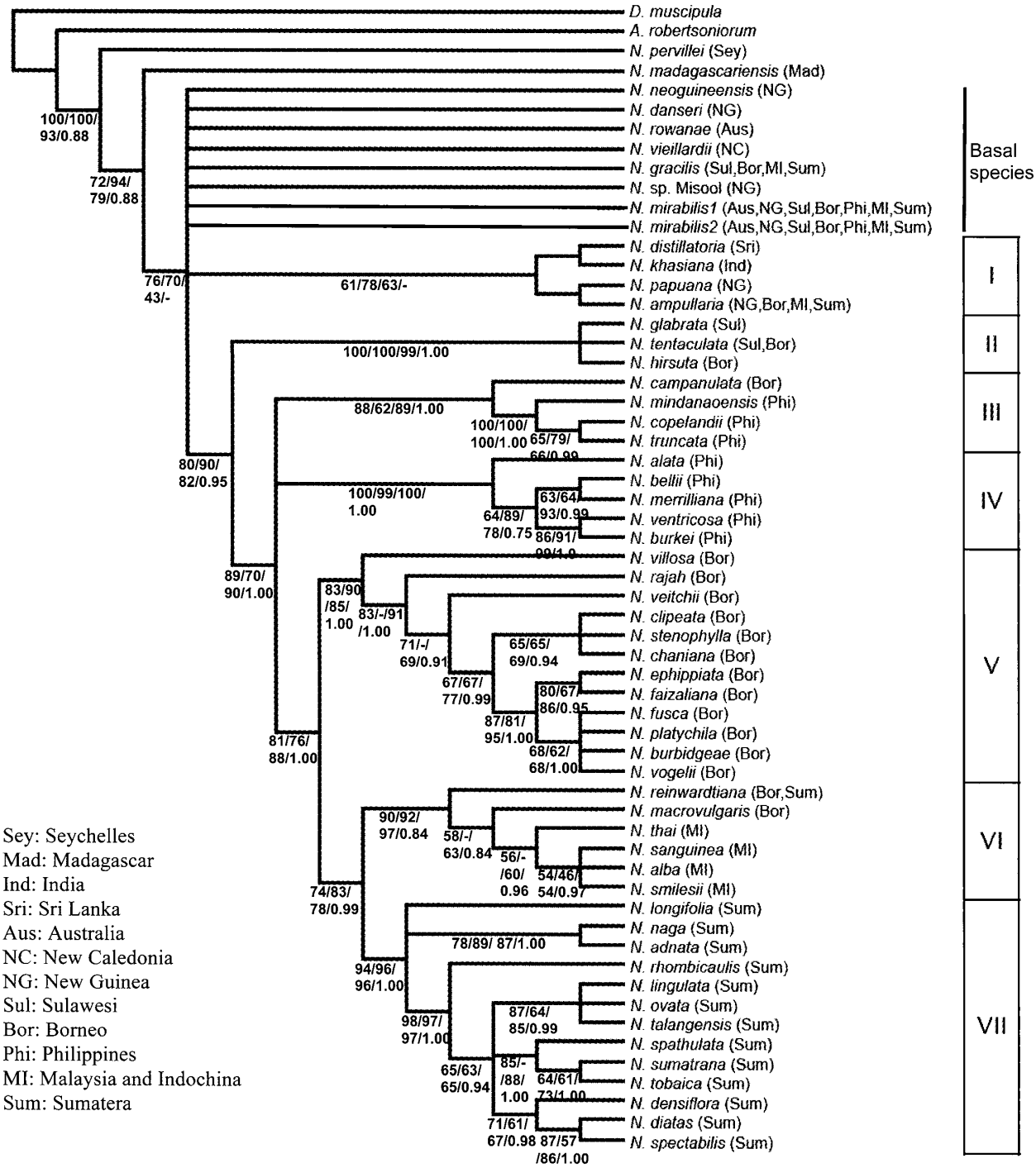

FIG. 1. Strict consensus tree derived from maximum parsimony analysis of ITS sequences of *Nepenthes* and outgroup taxa. Statistical support for each branch is shown below each branch with successive values of MP/NJ/ML/Bayesian. The minus signs (-) on the successive statistical values indicate different topologies or polytomies. Additional information on the distribution area of a the species is given as an abbreviation next to the taxon name.

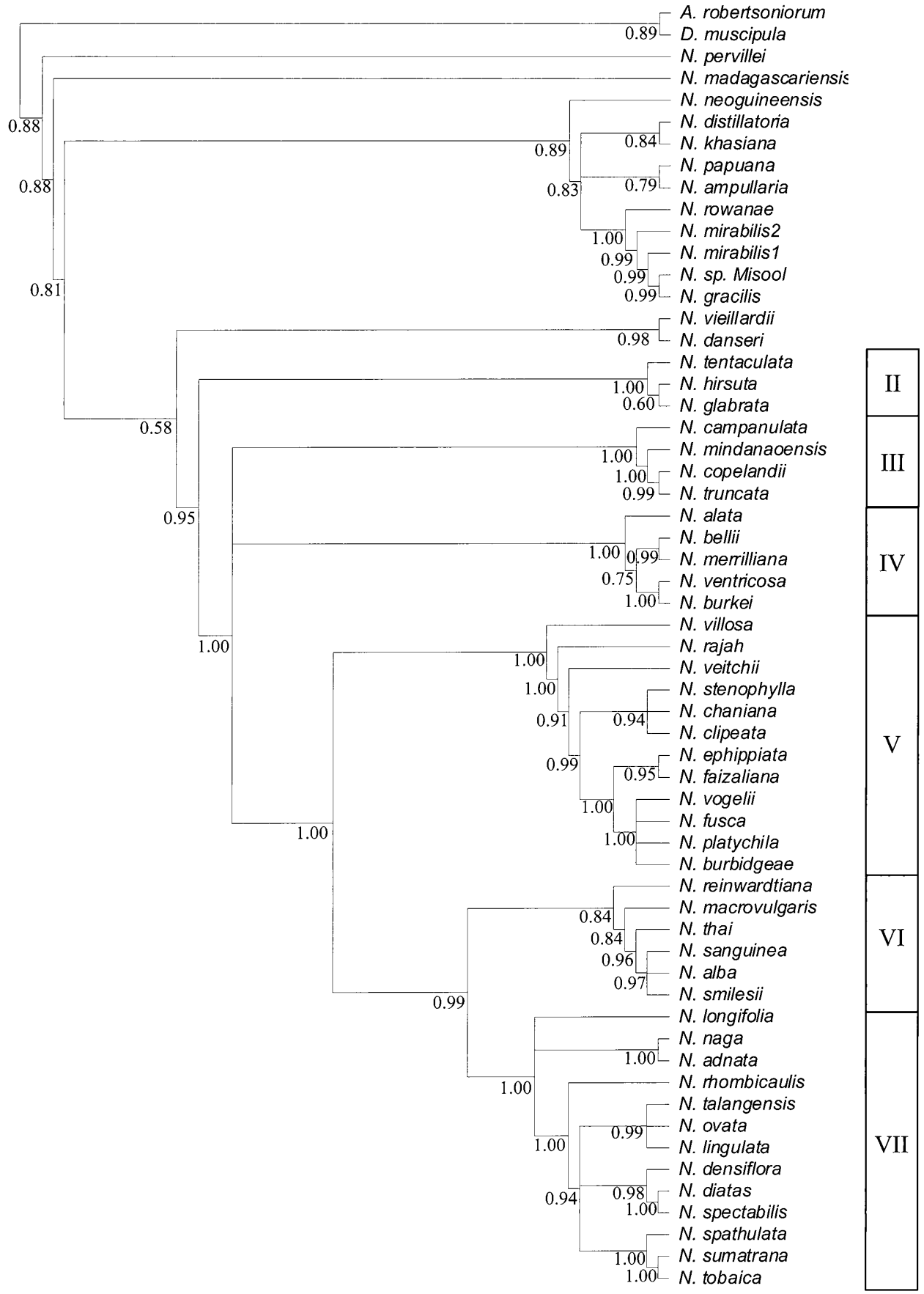

FIG. 2. The 50% majority-rule consensus tree derived from the Bayesian analysis of ITS sequences for *Nepenthes* and outgroup taxa. Numbers below branches are Bayesian posterior probabilities.

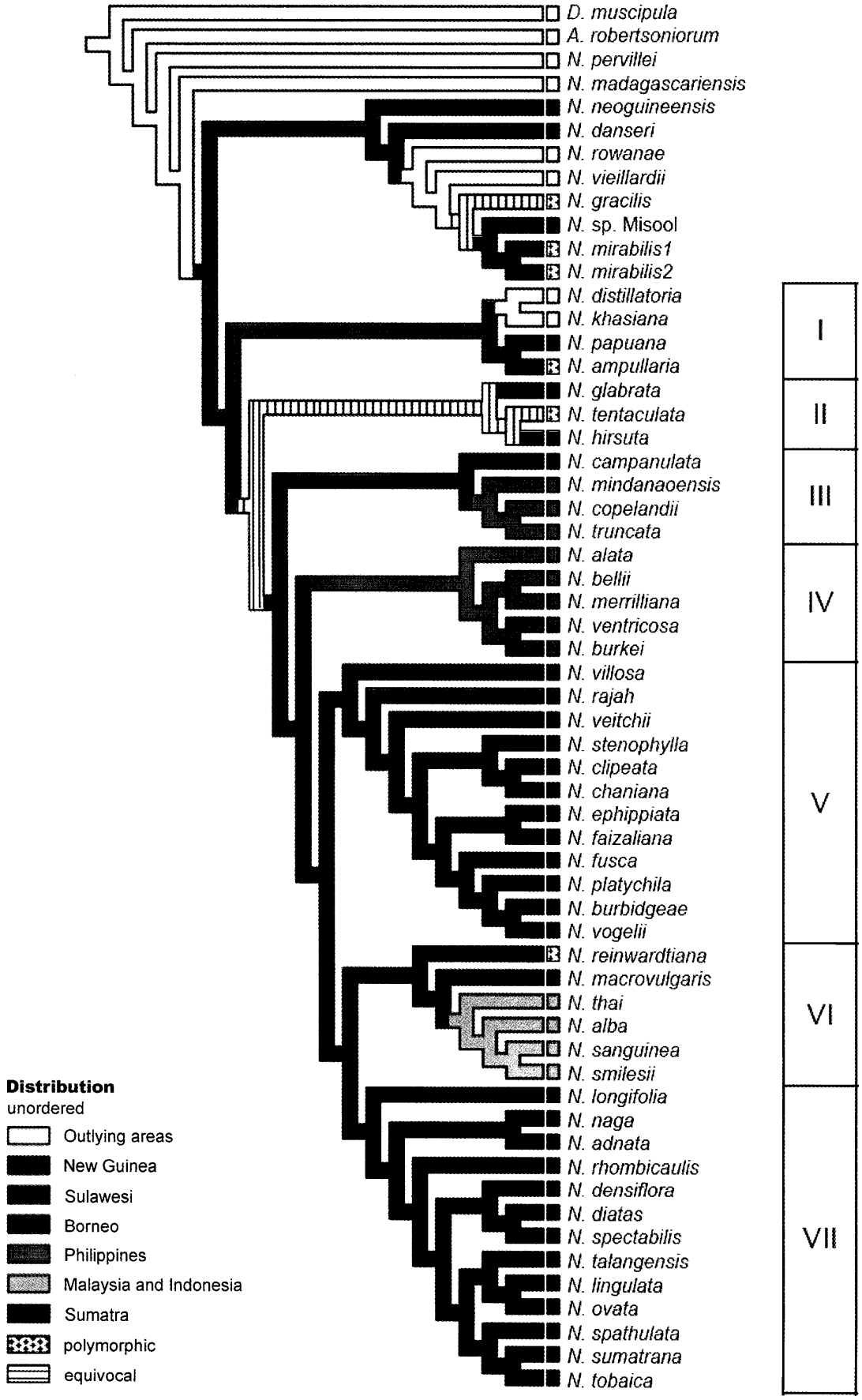

FIG. 3. Character state reconstruction of *Nepenthes* for distribution areas based on 1 of 324 MPTs using MacClade ver. 4.06 with ACCTRAN optimization. The squares next to the taxon name indicated subclades I–VII.

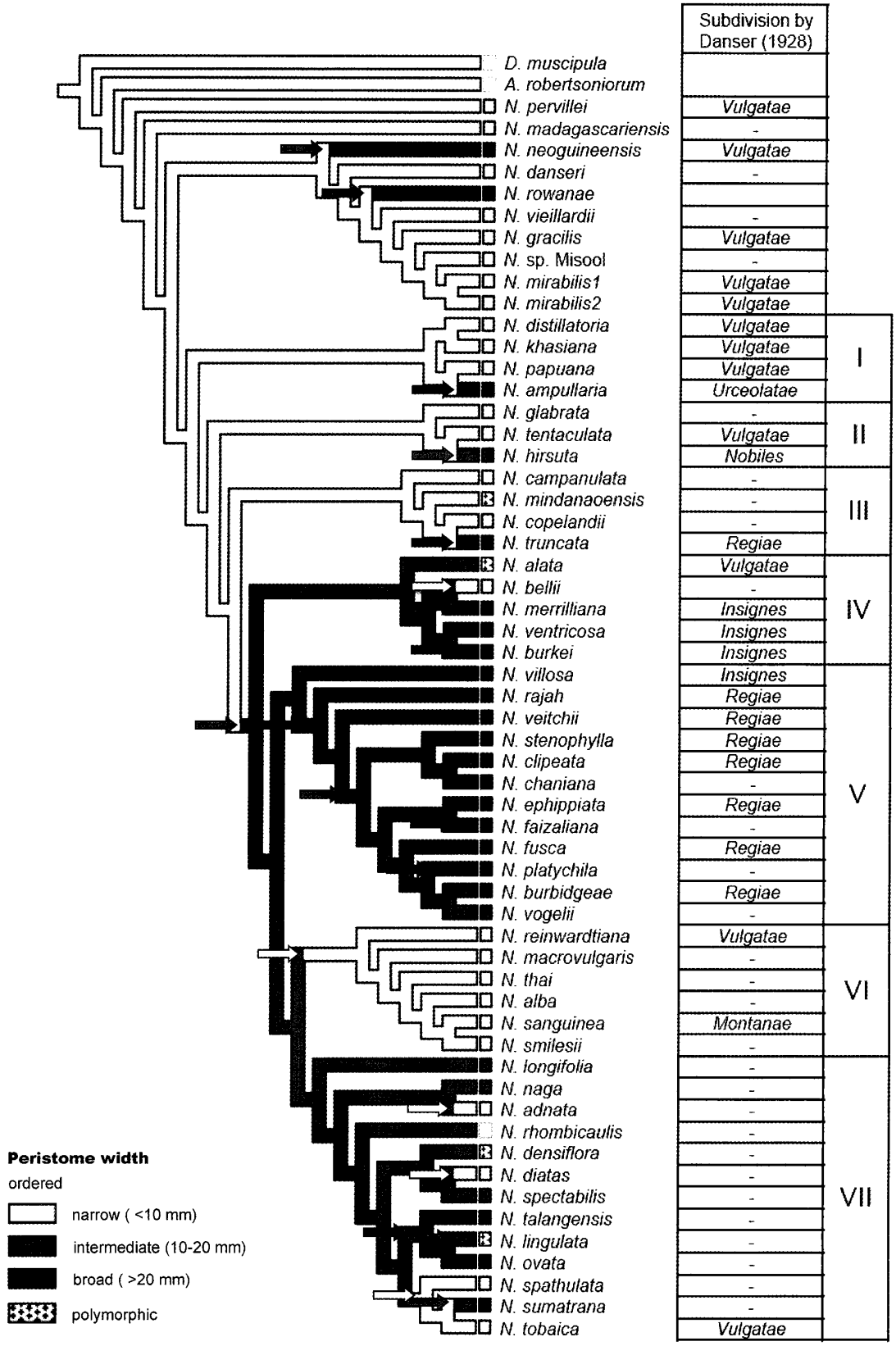

FIG. 4. Character state reconstruction of *Nepenthes* for peristomes based on 1 of 324 MPTs using MacClade ver. 4.06 with ACCTRAN optimization with comparison to the classification system of *Nepenthes* by Danser (1928), which is indicated by the squares containing group names next to the taxon names. The *arrows* indicate repeated evolution of narrow, intermediate, and broad peristomes. We recognized three character states of the peristomes: narrow (<10 mm), intermediate (10–20 mm), and broad (>20 mm). The character state for each species was obtained from the descriptions by McPherson (2009).

Java.

Seven species were indicated as basal polytomies on the MP phylogenetic tree, including two nonendemic species (*N. mirabilis* and *N. gracilis*) distributed across Borneo, Sumatra, Sulawesi, Peninsular Malaysia and Indochina and the Philippines, New Guinea, and northern Australia for *N. mirabilis*; a species from Misool Island located near the west coast of New Guinea (*N.* sp. Misool), two species from New Caledonia (*N. vieillardii*) and Australia (*N. rowanae*), and two species from New Guinea (*N. neoguineensis* and *N. danseri*), and Waigeo Island located near the northwest coast of New Guinea (*N. danseri*).

Character state reconstruction

The character state reconstructions of *Nepenthes* for distribution areas and peristomes, based on one of the 324 MPTs are presented in Fig. 3 and Fig. 4, respectively. Figure 3 shows the evolutionary trends of distribution areas of *Nepenthes*, which exhibits the radiation of *Nepenthes* on some islands and the migration of *Nepenthes* to adjacent islands or to mainland of Southeast Asia. Figure 4 shows the evolutionary trends of the peristomes of the upper pitchers and a comparison of the characteristics of the peristomes with the classification system of the genus by Danser (1928).

Discussions

Phylogenetic relationships in the genus Nepenthes

In our study of ITS DNA analysis (Fig. 1), we obtained phylogenetic trees with relatively higher bootstrap supports and Bayesian posterior probabilities than in previous studies employing the DNA region in the chloroplast genome (Meimberg *et al.* 2001) or the coding region of the nuclear genome (Meimberg & Heubl 2006), except for the basal positions of the trees. It is advantageous to use nuclear ITS regions for phylogenetic analysis in angiosperms.

Nepenthes pervillei was found to be the most basal taxon within the genus. It can be distinguished from all other species by its seeds, which lack the appendages typical of most species of *Nepenthes*. In addition, *N. pervillei* also has some unusual characteristics not found in other species of *Nepenthes*, such as nontwining tendrils that only slightly emerge from the upper pitcher; similar upper and lower pitchers; black, short, ovoid, truncate seeds and obconic fruit (Meimberg *et al.* 2001, McPherson 2009). The basal position of *N. pervillei* was consistent with reports from previous studies of the phylogeny of *Nepenthes* using the *trn*K intron (Meimberg *et al.* 2001, Meimberg & Heubl 2006, Meimberg *et al.* 2006). The second basal taxon in our study, *N. madagascariensis*, was also consistent with the *trn*K intron phylogeny of *Nepenthes* (Meimberg *et al.* 2001, Meimberg *et al.* 2006), but in a subsequent study of the phylogeny of *Nepenthes* using PTR1, *N. madagascariensis* formed a clade together with an Indian species, *N. khasiana*, and a Sulawesian species, *N. tomoriana* (Meimberg & Heubl 2006).

In our study, all the New Guinean species were included with the basal species and in subclade I with species from outlying areas, including Australia (*N. rowanae*), India (*N. khasiana*), and Sri Lanka (*N. distillatoria*), and the widely distributed *N. ampullaria*. The positions of the New Guinean species *N. papuana* in the same subclade as species from India (*N. khasiana*) and Sri Lanka (*N. distillatoria*) differ from previous studies of the *trn*K intron phylogeny of *Nepenthes*, where the Indian species (*N. khasiana*) was recognized as the third basal branch. The positions of the widely distributed *N. gracilis* and *N. mirabilis* as basal species, and *N. ampullaria* in subclade I of the tree topology as determined in our study were also different from reports in previous studies of the *trn*K intron phylogeny of *Nepenthes*, where these three widely distributed species were included in the same subclade as the Bornean species (Meimberg *et al.* 2001, Meimberg & Heubl 2006, Meimberg *et al.* 2006).

Despite the different positions of some species in subclade I and the basal species with previous studies of *Nepenthes* phylogeny, these species share similar morphological characteristics including acute leaf apex, orbicular or partly or-

bicular pitcher lids, and partly cylindrical lower pitcher form, except for *N. ampullaria*, which has a distinctive urceolate shape (McPherson 2009). Moreover, the pitcher morphology of *N. mirabilis* was shared by three species from outlying areas (*N. khasiana*, *N. distillatoria*, and *N. vieillardii*), which similarly lack putative apomorphic characteristics (McPherson 2009).

The positional differences of species between the present ITS analysis and previous studies using *trn*K intron and PTR1 (Meimberg *et al.* 2001, Meimberg & Heubl 2006, Meimberg *et al.* 2006) may be due to differences in the markers used. ITS is located in the nuclear genome (Baldwin *et al.* 1995), whereas the *trn*K intron is located in the chloroplasts, which are maternally inherited and may lead to the chloroplast capture phenomenon (Soltis & Kuzoff 1995). This phenomenon is considered the main reason for the incongruence of nuclear markers and plastid phylogenies (Meimberg & Heubl 2006, Tsitrone *et al.* 2003). In contrast, ITS has another weaknesses for estimating phylogeny. ITS is included in the rRNA precursor transcript. Genes encoding rRNA and spacers occur in tandem repeats that are thousands of copies long and polymorphisms in their sequences are often observed (Wendel *et al.* 1995a). Indeed, in the species of *Nepenthes* examined, we detected polymorphisms in seven species when using the PCR protocol with annealing temparature at 57°C. It is critical to decide which is an orthologous sequence for reconstructing the phylogenetic trees. It is possible that the incongruence of phylogenetic tree topologies is due to using paralogous ones. The concerted evolution (Wendel *et al.* 1995a) and intergenomic introgression (Wendel *et al.* 1995b) of the nrDNA genes may also have affected this incongruence. In the case of PTR1 (Meimberg & Heubl 2006), the gene is located in the nuclear genome, the same as the ITS. The incongruence between ITS and PTR1 trees may be due to amplification of paralogous sequences. As mentioned above, ITS sometimes shows polymorphisms, and PTR1 also exhibits the possibility of paralogous genes, since *Nepenthes* is suspected to be of polyploid origin (Meimberg & Heubl 2006). Lineage sorting is also considered to be a candidate for this incongruence. In addition to the incongruence discussed above, the basal region of our trees was not resolved well, and it is highly desirable to examine the phylogeny of *Nepenthes* using other DNA regions.

Phytogeography of Nepenthes *in Southeast Asia*

Meimberg *et al.* (2001) suggested the scenario that *Nepenthes* colonized Southeast Asia from an ancient Indian stock, first to the Malay Peninsula, and subsequently to Indochina and the Malay Archipelago. Their results showed the Indian species to be sister to the Southeast Asian species, although they did not reach a final conclusion. Our results show the Indian and Sri Lanka species forming a clade with the New Guinean *N. papuana* and with the widely distributed *N. ampullaria* and does not support the suggested scenario of Meimberg *et al.* (2001). In addition, three other New Guinean species, *N.* sp. Misool, *N. neoguineensis*, and *N. danseri*, were included in the basal polytomies with other widely distributed species, i.e., *N. gracilis* and *N. mirabilis*. The distribution area reconstruction on one of the 324 MPTs (Fig. 3) shows that these three widely distributed species may have expanded from east to west in Southeast Asia (east to west hypothesis). Of course, due to the polytomy of the basal species, the scenario of Meimberg *et al.* (2001) is also possible based on other MPTs. They considered that expansion of *Nepenthes* occurred from west to east in Southeast Asia, based on their tree topology, in which Indian species *N. khasiana* was sister to all of the Southeast Asian species. That topology was not supported by the PTR1 tree, where *N. khasiana* formed a clade with the species of Sulawesi and Madagascar (Meimberg & Heubl 2006). The east to west hypothesis was corroborated by similarities in morphology, altitudinal distribution and habitat types of the three widely distributed species, which were shared by species from New Guinea, Australia and New Caledonia (McPherson 2009).

The two species from Sulawesi, *N. glabrata* and *N. tentaculata* were included in subclade II together with *N. hirsuta*, a species endemic to

Borneo. This subclade is sister to subclade III–VII consisting of most species occurring in Borneo, Sumatra, the Philippines, Malaysia and Indochina. This tree topology and presences of *N. tentaculata* in Sulawesi and the Bornean Islands also suggests an important linkage for species of *Nepenthes* distributed across Southeast Asia from east to west (Fig. 3). The flora of Sulawesi is closely linked with the Australian-New Guinean phytogeographic region, which is indicated by the floral exchange between these three areas (van Welzen *et al.* 2011). Therefore, Sulawesi may have been a bridge for the migration of *Nepenthes* from east to west.

The distribution area reconstruction of *Nepenthes* (Fig. 3) suggests that *Nepenthes* diverged in Borneo, resulting many local species and could have also migrated to adjacent areas. *Nepenthes* probably migrated from Borneo to the Philippines. This scenario is supported by the presence of *N. campanulata* in the basal position of subclade III, which includes Philippine species. In addition, subclade IV consisting of Philippines species was included in clades (subclade III–VII), which is sister to subclade II consisting of species from Borneo and Sulawesi. This scenario also differs from that of Meimberg *et al.* (2001), which suggested migration from the Philippines to Borneo. In subclade VI, the two Bornean species were located in basal positions as sisters to the Indochina species and to all species of subclade VII that occur on Sumatra. Our results therefore suggest an ancestral species migrated from Borneo, diverged in Sumatra, then subsequently migrated to Java (*N. spathulata* is also known to be distributed in Java). It can be assumed that Borneo was a secondary center of diversification for *Nepenthes*, allowing species of *Nepenthes* to radiate within the Sunda Shelf region.

The scenario for *Nepenthes* expansion estimated from our molecular analysis based on ITS differs from that of Meimberg *et al.* (2001). These two scenarios were based on results from different DNA regions located in different genomes and may be due to the characteristics of the different DNA regions used in the phylogenetic analyses. The species examined in both analyses also differed, possibly affecting the tree topologies. Because the phylogenetic analysis based on ITS has weaknesses, especially in discriminating between orthologs and paralogs, it is highly desirable to base phylogenetic reconstructions on other DNA regions and by using additional species of *Nepenthes*.

Morphology of the peristome

Some morphological characteristics in the species of *Nepenthes* are polymorphic, and each of them has more than one form or shape within a species, making it difficult to evaluate the evolutionary trends in morphological characteristics. In this study, we tried to analyze some morphological characteristics, such as indumentum, inflorescence, lamina, leaf apex and base, lower and upper pitcher forms and the lid and peristome of the pitcher. We found the peristome of the upper pitchers to be consistent, except in *N. mindanaoensis*, *N. alata*, *N. ovata*, and *N. densiflora*. The peristome of the upper pitchers were relatively well correlated with the grouping of *Nepenthes* into seven subclades.

Figure 4 shows the evolutionary trends in the peristomes. The peristomes of the two basal species, as well as in most species in the basal polytomies and subclades I, II, and III, were narrow, suggesting that the narrow peristome is the plesiomorphic state in *Nepenthes*. In addition, all species in subclade VI produced narrow peristomes. Whereas the Bornean species in subclade V and most species in subclade IV and VII, as well as *N. ampullaria* in subclade I and two basal species (*N. neoguineensis* and *N. rowanae*), had intermediate and broad peristomes. The intermediate and broad peristomes likely evolved at least seven or eight times, respectively, whereas narrow peristomes evolved at least five times after the widening of the peristome. The findings suggest repeated evolution of narrow, intermediate, and broad peristomes.

In the taxonomic study by Danser (1928), narrow peristomes were considered to be ancestral in the *Vulgatae* group, which is concordant with our results, suggesting that *N. pervillei* and *N. madagascariensis* are basal species and that the

tree topology of subclade I and the basal polytomies consist of three species from the outlying areas (*N. distillatoria*, *N. khasiana*, and *N. vieillardii*), two New Guinean species (*N. danseri* and *N. papuana*), and two widely distributed species (*N. gracilis* and *N. mirabilis*). All of these species were assigned by Danser (1928) to the *Vulgatae* group (Fig. 4). Although *N. neoguineensis* in the basal polytomies produces intermediate peristomes, Danser (1928) assigned it to the *Vulgatae* group. Other species with narrow peristomes from three different subclades were also assigned by Danser (1928) to the *Vulgatae* group. Since representatives of the *Vulgatae* group are scattered in different subclades (Fig. 4), the group with narrow peristomes is obviously polyphyletic.

Intermediate peristomes can be defined as one of the key characteristica of the *Nobiles* group, while broad peristomes can be defined as a characteristic of the *Regiae*, *Insignes* and *Urceolatae* groups (Danser 1928). Since representatives of the *Insignes*, *Regiae*, and *Nobiles* groups occur in different subclades (Fig. 4), the three groups that share intermediate and broad peristomes are obviously polyphyletic. Judging from the tree topology (Fig. 4), the evolutionary trends in the character states of the peristomes reveal the limitations of Danser's classification of *Nepenthes*.

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